

Mineralisation in the teeth of the limpets *Patelloida alticostata* and *Scutellastra laticostata* (Mollusca: Patello gastropoda)

Katherine J. Liddiard^A, James G. Hockridge^B, David J. Macey^{A,C}, John Webb^A and Wilhelm van Bronswijk^B

^ADivision of Science and Engineering, Murdoch University, Murdoch, WA 6150, Australia.

^BSchool of Applied Chemistry, Curtin University, Bentley, WA 6102, Australia.

^CTo whom correspondence should be addressed. Email: d.macey@murdoch.edu.au

Abstract

The sequence and ultimate pattern of mineralisation in the major lateral radula teeth of two species of limpet, namely *Patelloida alticostata* and *Scutellastra laticostata* (Mollusca: Patello gastropoda), have been elucidated using energy dispersive, Raman and infrared spectroscopies. In both species, iron is the first element infiltrated into the teeth and, in the form of goethite, occupies the posterior cutting surface of the tooth cusps, whereas silica mineralises the rest of the tooth. The first onset of mineralisation, as judged by the initial influx of iron, occurs in the junction zone, the region separating the tooth cusp from the tooth base. Although differences between the two species do exist, the general pattern of tooth mineralisation is very similar, suggesting that the mineralisation of iron and silica is a very ancient character, both in this group and among molluscs as a whole.

Additional keywords: biomineralisation, goethite, iron, mollusc, silica.

Introduction

Molluscan teeth have long been a source of investigation and inspiration for biologists and chemists alike owing to the presence, in many species, of bioinorganic deposits (Lowenstam and Weiner 1989). These deposits, or biominerals as they are known and which are used to strengthen the teeth, range from the widespread hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) found in the teeth of many molluscan groups, to magnetite (Fe_3O_4), which is found only in chitons (Lowenstam 1962a; Kim *et al.* 1989; Macey and Brooker 1996). One of the more widespread molluscan groups is the limpets (order Patello gastropoda), which are common in the intertidal region throughout the world. In the limpets, as well as organic components, the major lateral teeth can contain up to 12% ferric oxide, chiefly as goethite ($\alpha\text{-FeOOH}$), 7%–16% silica in the form of hydrated amorphous opal ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$) and small amounts of calcium (Jones *et al.* 1935; Lowenstam 1962b; Runham *et al.* 1969; Grime *et al.* 1985; Burford *et al.* 1986; Mann *et al.* 1986; Lu *et al.* 1995). In limpets, as in most molluscs, the teeth are arranged in rows along a tongue-like organ, the radula. Because teeth at the mature end of the radula are lost continually by wear and breakage during feeding, they need to be replaced and, thus, the radula is composed of a sequence of teeth in various stages of mineralisation (Runham and Thornton 1967; Mann *et al.* 1986). Although several studies of the mineralisation of limpet teeth have been undertaken, they have been limited, in the main, to either bulked samples or individual teeth from which the various components have been extracted (see, for example, Mann *et al.* 1986). Recent advances in techniques now permit a detailed analysis to be conducted of the mineralisation process *in situ*, allowing a description to be given of both the process of mineralisation and the nature of the materials involved. The two limpet species investigated in the present study, namely *Patelloida alticostata* and *Scutellastra laticostata* (previously

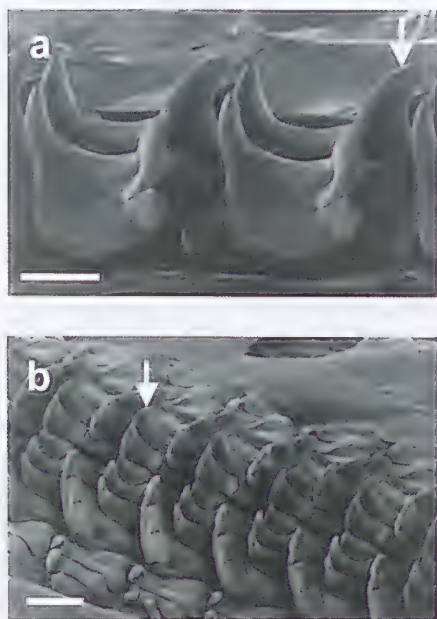


Fig. 1. Scanning electron microscope images of the radula of (a) *Patelloida alticostata* (arrow = posterior cusp of the tooth; scale bar = 100 μ m) and (b) *Scutellastra laticostata* (arrow = an interior major lateral tooth; scale bar = 200 μ m).

Patella laticostata), were chosen because they are representative of the extremes of limpet speciation (Lindberg 1998).

Materials and methods

Patelloida alticostata specimens were collected from limestone rocks in the splash zone at low tide in the Perth metropolitan area (32°S, 116°E), whereas *Scutellastra laticostata* specimens were collected from granite rocks at Bunker Bay (34°S, 115°E), also at low tide. Following collection, animals were placed in fresh seawater and transported immediately back to the laboratory, where the radula was dissected out. After removal, the radulae were placed in a solution of 4% NaOCl to remove any contaminating organic material. Once clean, the radulae were then positioned individually between glass slides to keep them flat during subsequent processing and placed directly into 30% ethanol. Radulae were examined at this stage using light microscopy to determine their overall structure, the number of tooth rows and the stage of tooth mineralisation.

The teeth of *P. alticostata* are arranged in a series of double rows with approximately 82 rows of teeth per radula. Each of the major lateral teeth, and there are only two per row, consists of a single anterior and posterior cusp connected by a single tooth base and flanked by a small marginal (Fig. 1a). In contrast, the larger *S. laticostata* possesses a much longer radula of approximately 185 tooth rows, the structure of which comprises one central unicuspid tooth flanked by a pair of unicuspid inner lateral teeth. These are followed by a tricuspid outer lateral tooth that is set behind the inner row, with an additional three, unicuspid marginal teeth set further out again in the radula (Fig. 1b). Light microscopy revealed that, in both species, only the lateral teeth were mineralised, as shown by the presence of a brown colouration, and, thus, only these teeth were subjected to analysis. In both species, initial elemental mapping was followed by a series of spot analyses obtained in various regions around the tooth cusp/s, in the junction zone that separates the tooth cusp from the tooth base and in at least three areas in the base.

For scanning electron microscopy, individual radulae were fully dehydrated through a graded series of alcohols. Those destined for morphological examination were dried further, using amyl acetate and critical point drying, mounted onto stubs using carbon tape, coated with carbon and gold, and examined using a Philips XL20 scanning electron microscope (SEM; Philips, Eindhoven, The Netherlands). Radulae destined for energy dispersive spectroscopy (EDS) were taken directly from 100% alcohol and sectioned crudely into lengths suitable for analysis. These sections were then placed flat into rectangular moulds and embedded in an Epon/Araldite mixture. So as to present the teeth in longitudinal section, the blocks were

P. alticostata*S. laticostata*

Fig. 2. Typical energy dispersive spectroscopy (EDS) qualitative elemental maps of the major lateral teeth of *Patelloida alticostata* (obtained at tooth row 51) and *Scutellastra laticostata* (obtained at tooth row 84). SE, Secondary electron image; Fe, iron map; Si, silica map.

then re-embedded, in the correct orientation, into aluminium discs. In order to obtain a flat and polished surface for analysis, these discs, together with control discs, were then ground using increasingly fine grades of silicon carbide paper, before being finally polished using a dry macroclean, microfibre cloth to reduce surface relief effects. Discs were cleaned between grades using an ultrasonic cleaner and 3% Extran 300 detergent (EMD Chemicals Inc., Gibbstown, NJ, USA). Discs were then rinsed with two changes of ultrapure water before being evaporative carbon coated. The EDS was performed using an Oxford LINK/ISIS system (Buckingham, UK) with a germanium window and a lithium drifted silicon detector attached to the SEM. Elemental mapping and spot analyses were performed at 20 keV using a spot size of 5 μm and a working distance of between 12 and 14 mm. Analyses were performed at regular intervals down the radula of both species, concentrating on regions in which light microscopy indicated rapid changes in mineralisation were occurring. Spot analyses were performed by acquiring at least three spectra from at least eight different regions of the tooth. These regions included the tooth tip, anterior and posterior sides of both anterior and posterior cusps, the junction zone and the tooth base. The EDS system was calibrated every 2 h using a cobalt standard.

Laser Raman spectroscopy was undertaken with an ISA Dilor Labram dispersive spectrometer (Longjumeau cedex, France) using both helium-neon (632.817 nm) and diode (783.532 nm) lasers. Unmineralised teeth were examined at various integration times before embedding, using the diode laser, so that interference due to the resin could be eliminated. Teeth from other stages of development were analysed following the resin embedding described above. Dark current background subtraction was performed on spectra from biological samples, with typical spectra acquisition times ranging from 1 to 6 h. Fourier transform laser Raman spectroscopy was also conducted on siliceous standards with a Bruker RFS-100 spectrometer (Sydney, Australia) using a neodymium:yttrium aluminium garnet (Nd:YAG; 1064 nm) laser and germanium diode detector. Spectra were obtained at 4 cm^{-1} resolution and were equivalent to those obtained with the helium-neon and diode lasers, confirming that these higher-frequency lasers did not degrade the siliceous material. The Raman spectra of the iron oxide and hydroxide standards used have been reported previously (Lee *et al.* 1998). α -Chitin (poly(*N*-acetyl-1,4- β -D-glucopyranosamine)) was obtained as a purified product (Sigma, St Louis, MO, USA), amorphous silica was synthesised using a sol-gel process (Munoz-Aguado and Gregorkiewicz 1997) and α -quartz and naturally occurring opaline silica (in the form of white sedimentary opal) were obtained from the Geology Department at Curtin University.

Infrared microscopy was conducted using a Bruker IFS-66 spectrometer fitted with a nitrogen-cooled EG&G Judson mid-infrared photoconductive mercury cadmium telluride detector system (MIR-MCT;

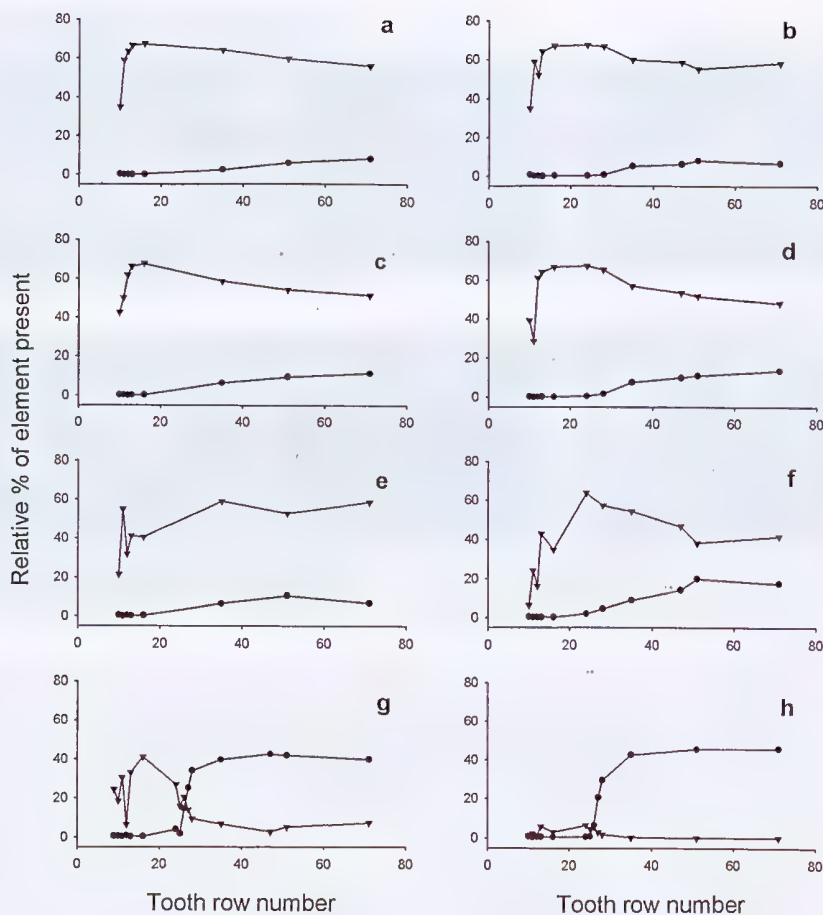


Fig. 3. Mean quantitative elemental spot analysis data of the major lateral teeth of *Patelloida alticostata* at various stages of development down the radula. (a) The tip of the anterior cusp; (b) the tip of the posterior cusp; (c) the posterior region of the anterior cusp; (d) the posterior region of the posterior cusp; (e) the anterior region of the anterior cusp; (f) the anterior region of the posterior cusp; (g) the junction zone; and (h) the tooth base. ▼, Percentage of iron relative to that of all other elements found; ● percentage of silica.

Montgomeryville, PA, USA) and a Bruker infrared microscope accessory and was collected at 4 cm^{-1} resolution. Synthetic iron oxides, amorphous silica and α -chitin standards were analysed as KBr discs with 512 scans being accumulated, whereas naturally occurring opaline silica and α -quartz standards were analysed by reflection, with an open aperture and 8192 scans being accumulated. Again, unmineralised teeth were analysed by infrared microscopy before the embedding process, so that interference from the resin could be eliminated, using an aperture selectively shaped to contain individual teeth, with 2048 scans being accumulated. Teeth from the later stages of mineralisation were analysed following resin embedding using reflection microscopy, with 8192 scans being accumulated.

Results

In *Patelloida alticostata*, qualitative EDS elemental mapping revealed that mineralisation, as evidenced by an influx of iron, commences in the junction zone at row 9, rapidly spreading to the posterior region of both the anterior and posterior tooth cusps by row 10

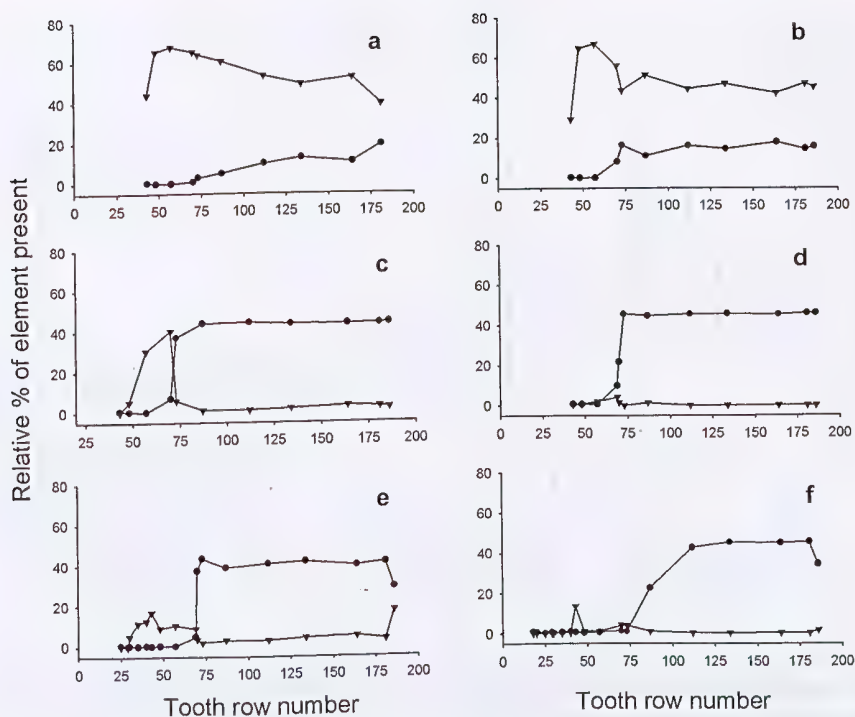


Fig. 4. Mean quantitative elemental spot analysis data of the major lateral teeth of *Scutellastra laticostata* at various stages of development down the radula. (a) The cusp tip; (b) the posterior region of the cusp; (c) the anterior region of the cusp; (d) the cusp core; (e) the junction zone; and (f) the tooth base. ▼, Percentage of iron relative to that of all other elements found; ●, percentage of silica.

and reaching high levels in these two regions by row 15 (an example of the type of map produced is given in Fig. 2). Quantitative multiple spot analysis confirmed the preliminary elemental mapping data, with initial levels of 22% iron being recorded in the junction zone, rising to 40% by row 15 before declining rapidly to less than 10% by row 28 (Fig. 3). In the tip and posterior regions of both the anterior and posterior cusps, iron levels peaked at just under 70% by row 12, declining only gradually down the length of the radula to approximately 60% in the tip and 55% in the posterior surface by the working teeth (Fig. 3). Although iron levels were also high in the anterior region of both the anterior and posterior cusps, the influx of this element occurred relatively slowly in the anterior region of the anterior cusp, not reaching a peak until row 35 (Fig. 3). Much higher iron levels were recorded earlier in the anterior region of the posterior cusp; however, these declined as maturation proceeded.

In contrast with iron, the influx of silica into the teeth of *P. alticostata* both occurred more slowly and was initiated later. Both EDS mapping and spot analyses (Fig. 3) showed that only small amounts of silica (less than 10%) were present in the posterior regions of both the anterior and posterior cusps, even in the fully mature teeth. Slightly more was found in the anterior cusps, but by far the largest amounts were found in the junction zone and the tooth base where silica predominated (Fig. 3). No evidence was found for any other elements, other than those associated with the organic components and the resin, at levels greater than 1%.

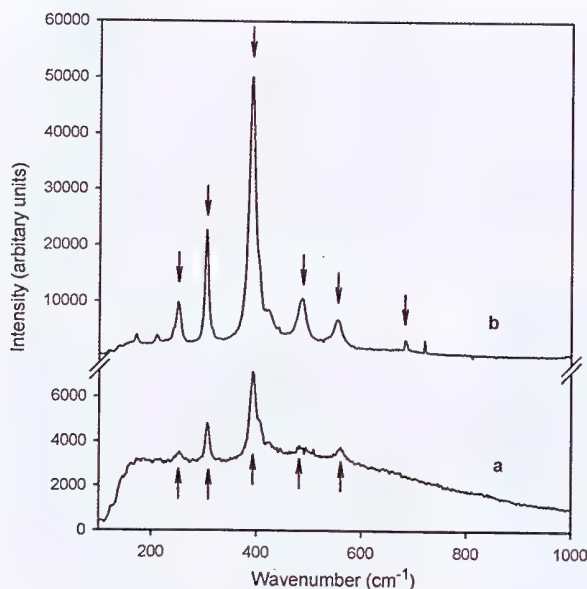


Fig. 5. Raman spectra of (a) an early mineralised major lateral tooth of *Patelloida alticostata* indicating the presence of goethite peaks (arrows) on a broad background, and (b) a late mineralised tooth of the same species now indicating the presence of strong goethite peaks (arrows) with relatively little background.

In *Scutellastra laticostata*, EDS mapping suggested that mineralisation followed a slightly different pattern (for an example of the type of map produced, see Fig. 2) with data obtained from spot analysis again confirming the initial impressions (Fig. 4). Thus, although the junction zone was again the first region of the tooth to show evidence of the influx of iron, in mature teeth this element was basically confined to the tip and posterior surface of the lateral teeth, with only small amounts being found in other regions of the tooth. The tip and posterior region of the tooth also contained up to 15% silica at the mature end of the radula. In marked contrast with the situation in the cusps of *P. alticostata*, where silica was virtually absent, the remainder of the cusp in *S. laticostata* was occupied almost entirely by silica, with only small amounts of iron being present. However, the composition of the junction zone and base was much more similar to that of *P. alticostata*, again being composed mainly of silica (Fig. 4). Low concentrations of calcium (approximately 3%) were also found early in the mineralisation process in the junction zone, although these declined rapidly (to less than 1%) as mineralisation progressed. Again, no evidence was found for any other elements, other than those associated with the organic components and the resin, at levels greater than 1%.

Raman microscopy of unmineralised teeth from both species revealed only the presence of α -chitin, with no evidence of the presence of iron oxides or siliceous compounds. However, the presence of weak, broad bands of ferrihydrite cannot be discounted. In both species, shortly following the initial deposition of iron, spectra obtained from the posterior surface of the tooth resolved a series of peaks characteristic of goethite (Fig. 5a). No evidence was found for the presence of any other minerals in any other regions of the tooth. As mineralisation progressed, the Raman goethite spectra became far more distinct and accounted for almost all the spectral bands observed (Fig. 5b). This form of spectroscopy indicated that the occurrence of goethite followed the initial deposition of iron very closely. Thus, for example, by tooth row 51 in *P. alticostata*, spectra positive for the presence of goethite were obtained from throughout the upper section of both the anterior and posterior cusps and in the surface of the 'saddle' region between the two cusps. Similarly, in

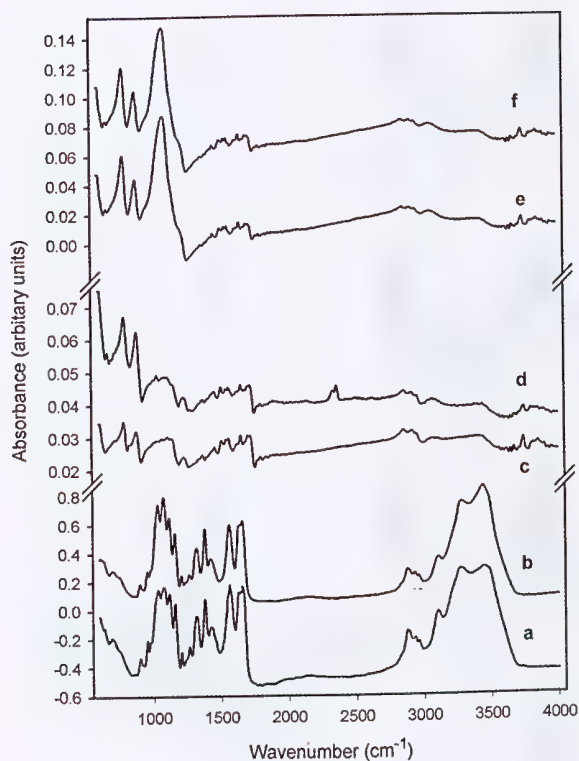


Fig. 6. Infrared spectra of the major lateral teeth of *Patelloida alticostata* (a, c, e) and *Scutellastra laticostata* (b, d, f); before the onset of mineralisation (a, b); at the onset of mineralisation (c, d); and later in the mineralisation process (e, f).

S. laticostata, by row 120, spectra strongly indicative of goethite were obtained from the tip and posterior surface of the cusp, whereas weak goethite spectra were also obtained from the upper anterior region of the cusp.

Infrared spectra of teeth from both species before the onset of mineralisation are presented in Fig. 6a,b. In both cases, the spectra are in close agreement with that of α -chitin, with the presence of distinct amide peaks in the spectral regions 1552 and 1616–1671 cm^{-1} being particularly useful for diagnostic purposes. There is no evidence of any contribution from ferrihydrite at approximately 1620 cm^{-1} at this stage of development. Early in the mineralisation process, the development of major absorption bands at 500–1000 cm^{-1} can be assigned to the presence of goethite. However, the presence of α -chitin (1250–1700 cm^{-1}) is still very obvious (Fig. 6c,d). Later in the mineralisation process, infrared spectra from both species were relatively unchanged in the region 500–1100 cm^{-1} , yet an intense peak at 1091 cm^{-1} has appeared that can be assigned to the Si-O-Si stretch of silica (Fig. 6e,f). The peaks due to α -chitin have also become relatively less intense. Spectra obtained from fully mature teeth (row 140 onwards) are dominated by the 1091 cm^{-1} silica peak, which has become very intense, whereas the peaks at 784 and 875 cm^{-1} have virtually disappeared (data not shown). The absorption bands due to α -chitin (1250–1700 cm^{-1}) have also completely disappeared, with a peak at 775 cm^{-1} becoming evident in both species. This later band can be assigned to the Si-O-Si bending vibration in opaline silica.

Discussion

The data presented in the present study allow, for the first time, a complete picture of the sequence of mineralisation in the major lateral teeth to be constructed in two limpet species,

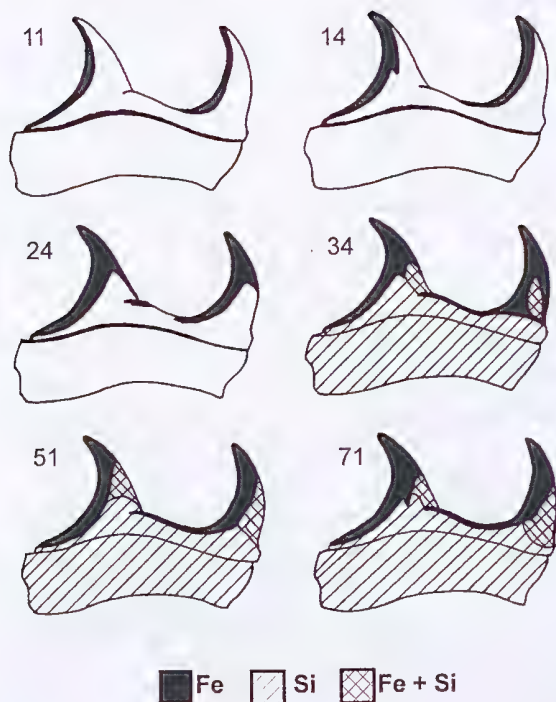


Fig. 7. Schematic representation of elemental inflow and subsequent mineral deposition in *Patelloida alticostata*. Tooth row numbers are indicated above the teeth.

namely *Patelloida alticostata* and *Scutellastra laticostata* (Figs 7, 8). Although specific differences do exist, the general pattern of tooth mineralisation is very similar, suggesting that the mineralisation of iron in particular and, to a lesser extent, silica is a very ancient character in the Patellogastropoda, and possibly in the Mollusca as a group (Cruz *et al.* 1998; Brooker and Macey 2001). However, tooth mineralisation in limpets, with its intermingling of complex mineral forms, is obviously very different from that in chitons, where different minerals are restricted to architecturally discrete compartments (Lowenstam 1967; Lee *et al.* 1998). In both limpet species, the initiation of the mineralisation process, as judged by the initial influx of iron, occurs in the junction zone, the region separating the tooth cusp from the tooth base (Figs 7, 8). In this regard, these two limpet species parallel the mineralisation process in the chitons *Cryptoplax striata* and *Acanthopleura echinata*, where mineralisation is also initiated in this region (Macey and Brooker 1996; Brooker *et al.* 2003). The presence, in this region, of high levels of iron and, later, silica, seen using EDS, without the subsequent large-scale deposition of minerals in the same area, also suggests that the junction zone acts as an initial reservoir, or conveyor, of material, allowing these elements to build up to a critical concentration before the onset of mineralisation proper in the upper regions of the posterior surface of the teeth and, thus, plays a very active role in the process (Macey and Brooker 1996; Brooker *et al.* 2003). Although these results would appear to be in contrast with those of previous authors who have suggested that mineralisation in limpets occurs initially in the tooth base, followed by the posterior surface of the tooth cusp (see, for example, Grime *et al.* 1985; Mann *et al.* 1986; Rinkevich 1993), the range of techniques used in the present study allows a far more detailed picture of both elemental and mineral deposition to be established. Although these

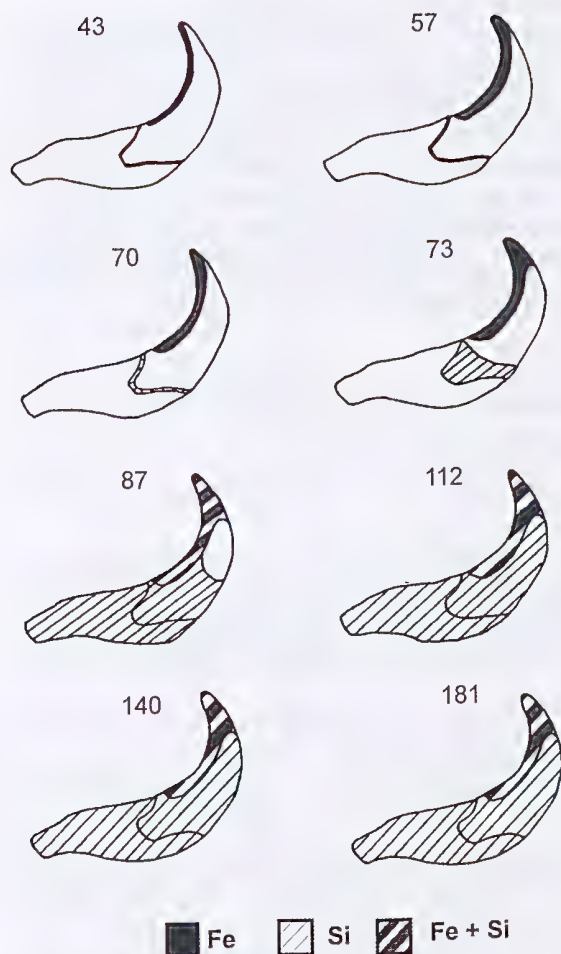


Fig. 8. Schematic representation of elemental inflow and subsequent mineral deposition in *Scutellastra laticostata*. Tooth row numbers are indicated above the teeth.

differences could be attributed to species variation, the deliberate choice in the present study of very divergent limpet species (Lindberg 1998), together with the parallel occurrence of the same phenomenon in chitons (Macey and Brooker 1996; Brooker *et al.* 2003), suggests that the initiation of the mineralisation process, where it occurs, through the junction zone is possibly widespread in molluscan teeth.

The very rapid spread of iron in the posterior surface of the tooth cusps of both species, following on from its initial appearance in the junction zone, may well be related to the need to allow for subsequent slow crystal growth in a favoured direction. Indeed, the presence of stoichiometric, well-ordered crystals in this region, in contrast with the superparamagnetic, poorly ordered microcrystals located within the tooth base, has been noted previously (Mann *et al.* 1986; St Pierre *et al.* 1986). In contrast, in both species both the deposition and mineralisation of silica occur much later in tooth development, possibly reflecting the less-ordered structure of the 'scrolls' formed (Mann *et al.* 1986). The presence in the tooth bases of large amounts of silica, presumably in the form of loosely aggregated scrolls, rather than crystalline goethite may well be due to the need for the base to be more flexible than the cusp, allowing for the impact of the tooth cusp striking the hard surfaces on which the limpets feed.

The relationship between tooth structure, in terms of the number of overall impact points and the degree of mineralisation has been well established across a wide variety of molluscan species (Steneck and Watling 1982). The reduced number of impact points in *P. alticostata* (four), compared with the far larger number in *S. laticostata* (10), is paralleled both by a far greater proportion of the tooth cusp being mineralised with iron in the former species and by the complete lack of silica in the posterior cusp surface of *P. alticostata*. All these factors suggest that the cutting surface of the major lateral teeth in *P. alticostata* is much harder than that of *S. laticostata*, indicating, in turn, that the two species have very different feeding habits, with *P. alticostata* penetrating into the substratum on which it is found to much greater degree than *S. laticostata* (Black *et al.* 1988).

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